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14. ABSTRACT

High intensity focused ultrasound (HIFU) has been combined with a Rose Bengal derivative (RB2) to provide a synergistic cytotoxicity requiring the presence of both ultrasonic cavitation and drug. In vitro tests have shown that a short treatment (less than 30s) of pulsed HIFU with power sufficient for cavitation destroys >95% of breast cancer cells in suspension with 15uM of the compound. Neither the pulsed HIFU nor the RB2 compound was found to have any impact on the viability of the cells when used alone. In vivo tests using these same cells growing as a xenograft in mice were also done. Using similar treatment parameters in vivo necessitated the use of ultrasound contrast agents to initiate cavitation. When this was done, we were able to demonstrate rapid tumor regression for combination pulsed HIFU and RB2 treatments. RB2 was delivered in large doses of 250 mg/kg i.v. without any adverse side effects. We believe it should be possible to develop this technology into an alternative safe and effective means of tumor ablation for breast cancer treatment.

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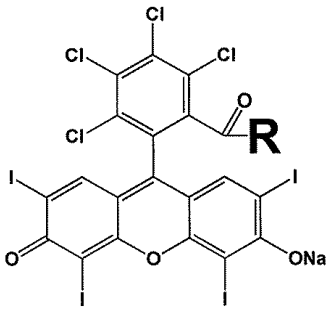
Introduction

High power ultrasound creates and interacts with microbubbles, causing extremes in pressure and temperature that can reportedly "activate" molecules known as "sonosensitizers". Called "sonodynamic therapy", this technique is often seen as the ultrasonic analog of the clinically tested photodynamic therapy, and historically researchers have used the same compounds for both therapies[1]. However, many of the problems associated with photodynamic therapy, including side effects resulting from exposure to ambient light, could be solved by treating with a compound that is activated by sound (a "sonosensitizer") but not by light. In our study, we have introduced and tested a potent new sono-sensitive compound based on Rose Bengal which is not photo-sensitive. This compound (RB2) was tested *in vitro* and *in vivo* in combination with cavitation driven by high intensity focused ultrasound (HIFU). Applying HIFU in pulsed mode (to avoid overheating) has shown promise in improving the penetration of large therapeutics into tumors. It is also capable of producing consistent cavitation activity even deep in the tissue. This project was designed to test whether using pulsed HIFU for delivery and activation of a sonosensitizer might result in an effective targeted chemotherapy that could be useful for treating breast tumors without the side effects associated with traditional untargeted chemotherapy or photodynamic therapy. The *in vitro* work consisted of looking for a synergistic cytotoxicity between RB2 and pulsed HIFU treatment of a breast cancer cell line. The *in vivo* studies were designed to test for systemic toxicity of the compound and synergistic anti-tumor effects when applied to a breast cancer xenograft model.

Body

Chemistry

The initial research plan called for the use a known porphyrin that was reportedly sonosensitive by not photosensitive. As it turned out, this compound, DCPH-P-Na(I), was not commercially available and would have needed to be custom produced. Dr. Tung, our chemistry expert consultant, advised that porphyrin chemistry was difficult and required expensive equipment beyond the scope of the project budget. He suggested that we instead modify another readily available photosensitive compound, Rose Bengal, as well as purchase a set of commercially available porphyrins for testing. In the end, we obtained hematoporphyrin IX (HP), mesoporphyrin IX (MP), protoporphyrin IX (PP), and isohematoporphyrin IX (IP) from Frontier Scientific, Inc. As well, we purchased Rose Bengal and from it produced two analogs in Dr. Tung's lab, labeled RB1 and RB2, for testing. The final compounds' chemical structures are shown in Fig. 1. RB is Rose Bengal. A carbon chain was added to form RB1, with increased affinity to the cell membrane. To form RB2, the active oxygen is replaced with nitrogen,



R: RB: -OH
RB1: -O-$(CH_2)_{12}$-CH_3
RB2: -NH-$(CH_2)_{12}$-CH_3

Rose Bengal

Fig. 1: Chemical structure of Rose Bengal analogs.

resulting in a molecule that should be insensitive to light. These modified compounds and their potential uses were covered under a provisional patent filing from late 2008.

In Vitro Studies

In vitro testing of the compounds began in September 2008 when the RB derivative became available. Fifteen milliliter polystyrene tubes were filled with 12 ml of 1% agarose (Invitrogen Corporation, Carlsbad, CA, USA). The tubes were sterilized using the UV lamp in the hood for 15 minutes. Since some compounds demonstrated poor solubility in PBS solution, all the compounds were dissolved in DMSO. MDA-MB-435s (or MDA-MB-231) human breast adenocarcinoma cells were obtained from American Tissue Culture Collection (ATCC, Rockville, MD, USA). The cells were grown at 37°C and 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation, Carlsbad, CA, USA) and antibiotics (100 U penicillin/ml and 100 µg streptomycin/ml; Invitrogen Corporation, Carlsbad, CA, USA). The day previous to the assay, 10 x 10^6 cells were seeded in 10 cm tissue culture plates and incubated over night. The day of the assay, the media was removed and the cells were washed twice with cold PBS. The cells were treated in serum free media with different concentrations of compound for 30 minutes. During this time the cells were kept in the incubator at 37°C and 5% CO_2. After the treatment, the cells were suspended in the same media and 10^6cells/ml and were aliquoted into the tubes (around 3 ml per tube). The tubes were covered with an "acoustic window", a film of 1 mil poly held with an appropriately sized o-ring. Great care was taken not to introduce or leave any air bubbles under the film. The tube was then suspended in a degassed water bath and treated with pulsed HIFU through the acoustic window. The treatment parameters were typically 1 Hz repetition rate, 50% duty cycle, 5 or 7 MPa peak negative acoustic pressure, for 5 minutes (later shortened to 30 seconds). The acoustic pressure and treatment length were varied during different studies. The transducer was an f=1annular array with a focal distance of 8 cm, operating at 1.4 MHz center frequency. The system was manufactured by Philips Research for research purposes (Unison, Philips Research, Briarcliff Manor, NY), and is limited by a maximum total acoustic output of 40 W (abou 8.6 MPa peak negative pressure). Targeting and cavitation monitoring was via ultrasonic b-mode imaging. After the ultrasound treatment, the cells were spun (1000 rpm for 5 minutes) to remove the media and re-suspended in fresh media with 10% FBS and antibiotics.

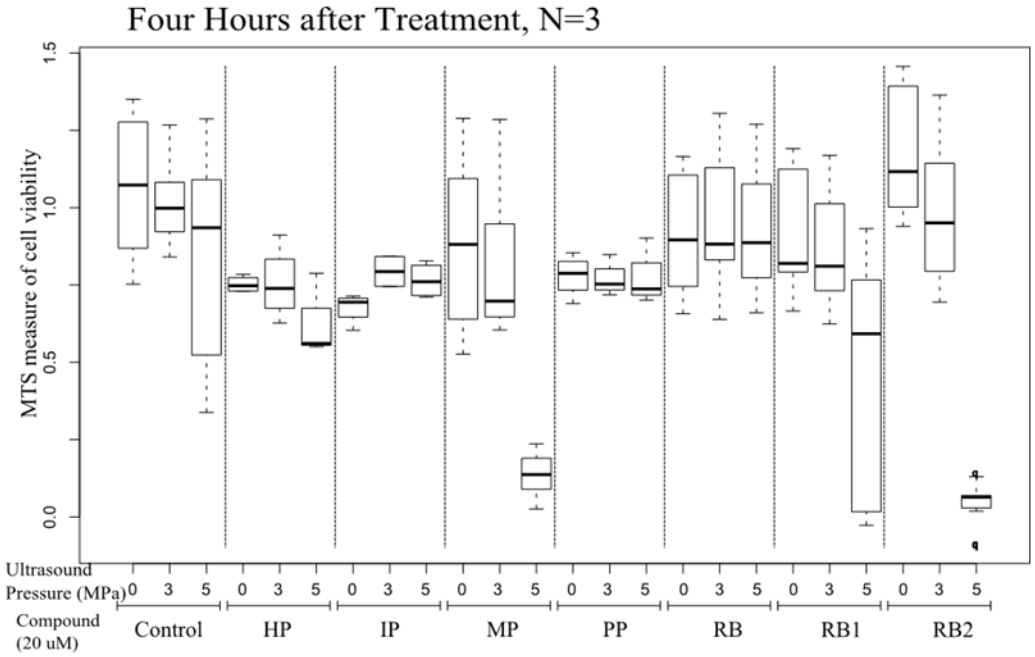


Fig. 2: Synergistic effect of pulsed HIFU and candidate sonosensitizers: porphyrins (HP, IP, MP, PP) and Rose Bengal derivatives (RB, RB1, RB2). Only RB2 is not a photosensitizer.

To assess cell viability, 15, 000 cells per well were seeded in a 96-well plate. The cell viability was assessed by two different methods: trypan blue dye exclusion and by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay . For the trypan blue dye exclusion, immediately after treatment the cells were diluted in 0.4% of trypan blue solution (Invitrogen Corporation, Carlsbad, CA, USA), loaded in a hemocytometer and counted under a microscope. The results are expressed as a percentage of unstained cells to total number of cells. The MTS assay was performed using a commercially available kit (Promega, Madison, WI, USA). Briefly, after plating the cells (15,000 cells/well), 20 µl of MTS solution was added to each well. The plates were incubated at 37°C and 5% CO_2 for 4 hours (time point, 1 hour after treatment). This procedure was repeated four hours after the ultrasound treatment (time point 4 hours), and in some cases, on the next day (24 hrs). After the incubation time, the absorbance at 492nm was measured using a FLUOstar OPTIMA microplate reader (BMG LABTECH Inc., Durham, NC). All measurements were taken in quadruplicate. The results are expressed as the percentage of cell viability of the treatments compare to the controls.

All tests were carried out with N≥3.

The first successful study compared all seven compounds and drug free control in combination with 0, 3, 5 MPa. These results immediately showed great promise, particularly for the RB2 compound (see Fig. 2). One of the more effective porphyrins, the HP, was also significantly toxic on its own, and was therefore used at higher dilutions than the other compounds. Histology demonstrated that the cell killing was by immediate lysis (Fig. 3) rather than apoptosis. As might be expected in this case, the only significant differences between data collected at 1, 4 and 24 hrs is attributable to cell growth in the control dishes.

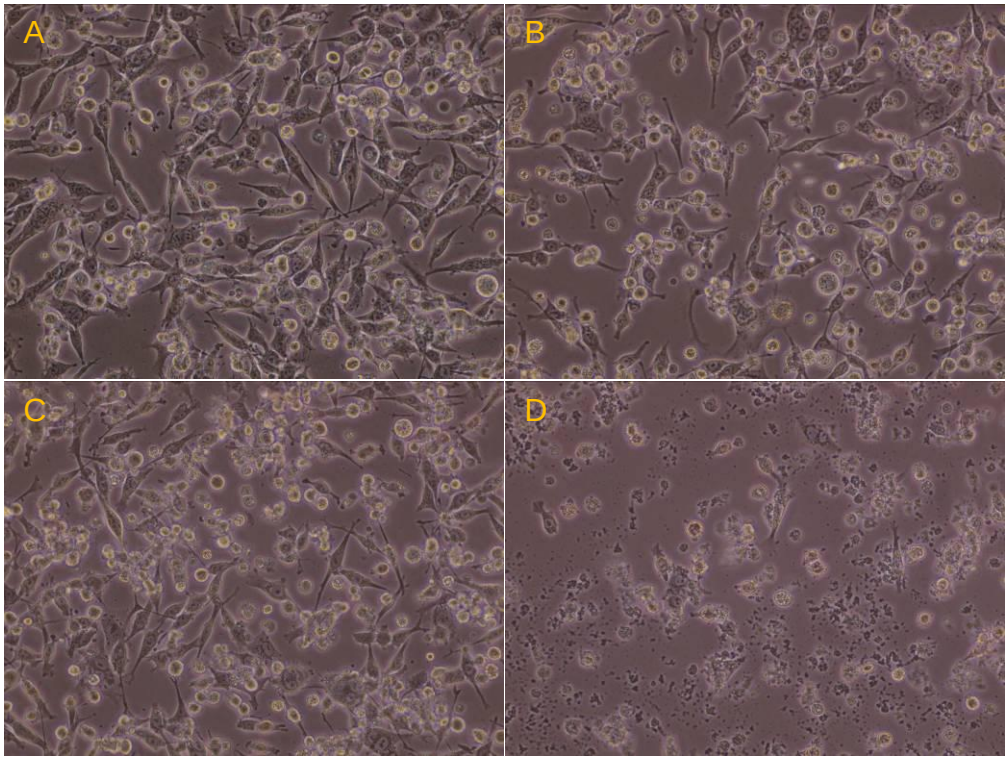


Fig. 3: Cells following treatment. A: Control; B: RB2; C: RB2+3MPa; D: RB2+5MPa

Following this success, we then went ahead with testing the RB2 and MP compounds at various doses, using an ultrasonic pressure of 5 MPa as a baseline treatment. Both the MP and the RB2 showed progressively more cell killing with higher dose, however, these studies were somewhat confounded by the variability from one tube to the next. This variability had to do with differences in bubble formation, and during this study, we established a clear link between bubble formation, assessed by the ultrasound imaging backscatter, and cell kill (Fig.). Hoping to control the variability in bubble formation, we did a series of tests with addition of pre-formed microbubbles (Optison, GE Healthcare), however, this did little to solve the problem. During one test the tubes were inadvertently switched from polystyrene to polypropylene, resulting in a noticeable change in bubble formation. It was also noticed that many the bubble in the polystyrene tubes tended to stick to the plastic walls. It thus became clear that the plastic in the tubes was not an inert bystander, but was influencing the tests by interacting with either the cavitation or the compound. Therefore, the plastic tubes were replaced by borosilicate glass test tubes (11mL, 15 x 85 mm) and the studies repeated and extended. This effectively reduced the variability in the results, however, in order for to achieve the same cytotoxicity, the baseline ultrasonic pressure had to be increased from 5 to 7 MPa in the glass tubes.

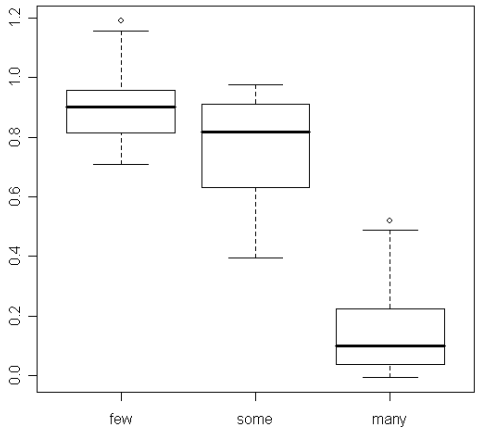


Fig. 4: Cell viability dependence on bubble activity.

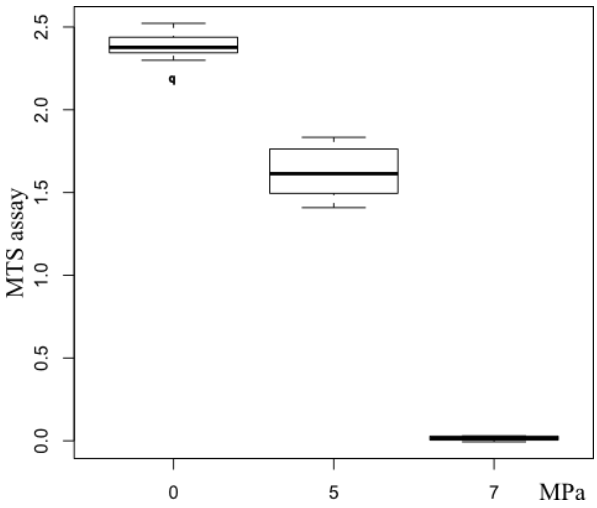


Fig. 5: Viability vs. ultrasound pressure; 20 uM RB2.

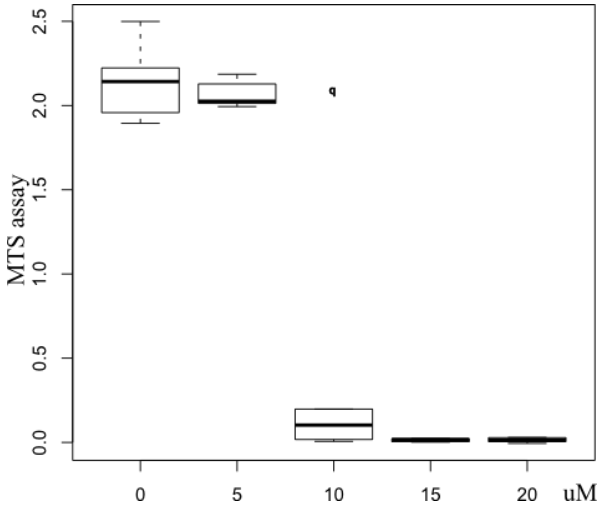


Fig. 6: Viability vs. RB2 dose; 7 MPa ultrasound.

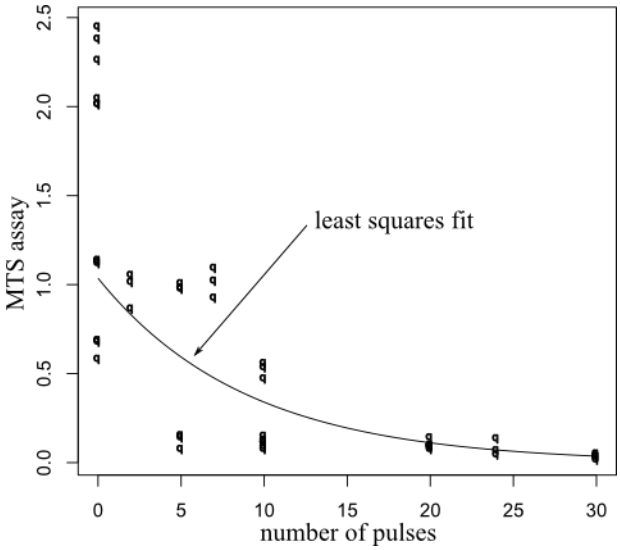


Fig. 7: Viability vs. time (number of pulses) for 20 uM RB2, 7MPa ultrasound.

Figures 5-7 show the results of studies in the glass tubes, using only the RB2 compound with varying ultrasonic peak negative pressure, drug dose, and treatment time. (The differing scales on the y-axis compared to Figs. 2 and 4 result from a different starting concentration of cells.) All of these factors are very significant ($p<10^{-12}$) and the synergy between the ultrasound treatment and the compound dose is clearly evident. Using the glass tubes greatly reduced the variation from tube to tube, although there remains a set of tests included in Fig.7 that appears "off". We suspect that this may have been due to poor targeting during the ultrasound treatments that resulted in only partial exposure to the beam. This occurred during a period when the ultrasound image guidance was being replaced and may not have been properly calibrated.

Based on the data in Fig. 7, it is possible to fit an exponential decay curve:

$$V(t) = Aexp(-\alpha t)$$

where *V(t)* is the viability, and *A* and α are parameters to be determined. This was done in the linearized form data using the least squares routine in R (R Foundation for Statistical Computing, Vienna, Austria). The resulting parameters are: ln(*A*)=0.04±0.15 (P=0.81) and α=0.1114±0.0098 (P=5.89e-15), with R^2=0.73. In effect, this means that with every pulse (at 1 Hz), 11% of the remaining cells are killed. Put another way, 30 seconds is sufficient to kill over 96% of the cells in solution.

In Vivo Studies

In vivo testing in mice began in late January 2009, as soon as we were convinced the compounds had potential. In keeping with the original statement of work, only the compound that was not photosensitive (RB2) was adopted for animal studies. MDA-MB-231 cells were inoculated in the posterior regions of both sides of female nu/nu mice and allowed to grow. After these tumors reached an appropriate size, the animals were (optionally) i.v. injected with the RB2 compound and Optison, and treated with pulsed HIFU. (The protocol also called for a "pretreatment" HIFU dose administered prior to the compounds to enhance their delivery. This option was not often used as it would have added significantly to the confusion surrounding the results, and likely would not result in any significant advantages for a small molecule such as RB.) The HIFU was administered by partially submerging the animal in a bath heated to 35-36 C. The ultrasound was then coupled from above using a waveguide of degassed water and was targeted via B-mode ultrasound imaging of the animal. Typical HIFU treatment parameters are 7 MPa peak negative pressure, 1 Hz repetition rate, with sonication locations arranged on a grid with 2 mm separation distance. The transducer focal zone is an ellipsoid of major and minor axes 7 mm and 1.5 mm respectively. Some of the earlier animals received a dose with 5% duty cycle and 100 pulses, but this was deemed too far removed from the in vitro parameters, so it was later changed to 20% duty cycles and 30 pulses. The 20% duty cycle was used rather than the 50% employed in the in vitro studies to avoid excessive heating. Optison was used in most animals, as we had already determined that the Unison system most likely couldn't provide enough power to directly induce cavitation in solid tissue. Unfortunately, Optison microbubbles are relatively large, and are quickly removed from the blood stream. To avoid complication due to timing the treatment, it was decided that in intra-tumoral injection would be best. Unlike the Optison, RB2 is a small molecule, and likely able to diffuse directly into tissues. Therefore we felt intra-veinous tail vein injections would work for this compound. To adhere as close to the in vitro results as possible, the highest dose was used, 20 μM of RB2 by volume of the mouse. This translates to 250 mg/kg, but despite the incredible dose, no adverse effects of the drug were ever observed in the absence of pulsed HIFU treatment.

Two major complications arose that slowed the pace of the in vivo work. First, the MDA-MB-231 cell line would only take well in animals inoculated when very young, less than about 8 weeks. Older animals would sometimes develop tumors easily, sometimes only very slowly, sometimes not at all. Of the first 15 animals inoculated, only 4-5 developed tumors of a size that was treatable. The second problem became apparent much later. Most of the early animals were treated when the tumors were 100 to 200 mm^3 in size, and their tumors taken for histology within 24 hrs. It was expected that if the gross effects observed in vitro were to translate to the animal model, it would be easily seen as large regions of necrosis/lysis with plenty of micro- or macro-hemorrhage. However, this type of pathology was clearly visible in many of the control tumors as well. Thus, even though there were some histological effects we believe may have been due to the treatment (see Fig. 8), the results are inconclusive. Also, because the tumors were already necrotic at this size, they quickly ulcerated if left to develop, and thus were difficult to enroll in a longitudinal therapeutic study.

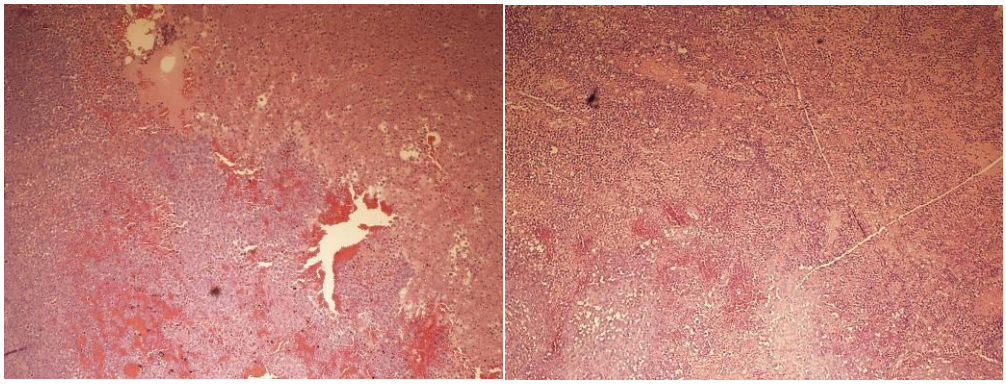


Fig. 8: Sonodynamically treated MDA-MB 231 tumor (left) and control (right).

Ultimately, our best results came from animals treated with undersized tumors of ~50 mm^3. In the few such animals we were able to treat prior to July 15, tumor regression was consistently observed (Fig. 9). Treatments with no RB2 injection (Optison i.t. only) appear to have a weaker effect. Additional animals treated since then have shown similar promising results. To really substantiate this effect, however, requires additional control experiment that have not yet been completed. Even then, however, some questions would undoubtedly remain. One problem with treating small tumors with HIFU is the skin effect. Since the transducer focus (length 7 mm) invariably passes through the skin, which absorbs ultrasound and heats more easily than the tumor tissue, we cannot be certain that this is not impacting the effect we see. A better (larger) tumor and animal model would improve this situation.

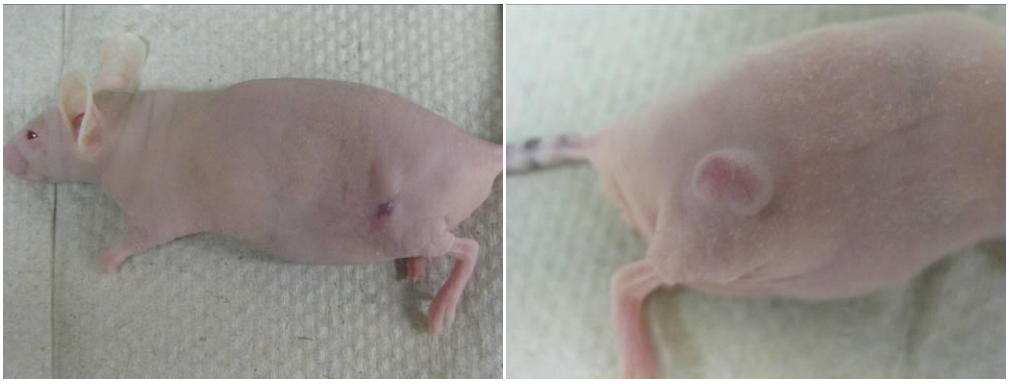


Fig. 9: Treated (left) and control (right) tumors, 10 days after sonodynamic treatment.

Due to the extra in vitro work that was needed, as well as the problems we encountered with the tumor model, a number of tasks on the statement of work were not completed within the one year time frame. These include a portion of Task 4 (rigorous study with a full set of controls), Task 5 (test of pretreatment for compound delivery) and Task 6 (test of singlet oxygen production). In some sense, Task 5 and Task 6 may be unnecessary, since there may be no need to use the pretreatment option, and it is doubtful that this type of massive cell lysis can be explained by singlet oxygen production. We intend to complete Task 4 in the next few months and we also plan to apply commonly used in vitro tests to check for singlet oxygen and free radicals production.

Key Research Accomplishments

- Created a novel sono-sensitive compound based on Rose Bengal to replace the DCPH-P-Na(I) compound that we were unable to obtain. Like the original compound, RB2 is based on a photosensitive drug, but was altered to:
    1. have greater affinity to the cell membrane, and
    2. have no light associated toxicity.
- Tested the new compound in vitro against breast cancer cell lines, using pulsed high intensity focused ultrasound for activation. Demonstrated strong synergistic effect, with the combination of compound and ultrasound cavitation killing over 95% of cells, while neither the drug nor the ultrasound alone showed any significant effects.
- Compared RB2 to various sonosensitive porphyrins mentioned in the literature.
- Found HIFU activation is in general more lethal than the activation reported in sonodynamic literature using unfocused ultrasound.
- Demonstrated that cavitation is required for this effect.
- Demonstrated that the method of killing is direct membrane disruption (lysis).
- Tried to duplicate these results in vivo to ablate a xenograft breast tumor with an i.v. tail vein injection of RB2 and direct i.t. injection of Optison followed by HIFU treatment to nucleate cavitation. Current results are promising but not yet conclusive.

Reportable Outcomes

1. Provisional patent on RB2 compound
2. Manuscript (in preparation) describing in vitro work
3. Manuscript (in preparation) describing in vivo work
4. NIH grant application (in preparation)

Conclusion

In the current study, we have tested a novel sonodynamic compound, derived from the photosensitizer Rose Bengal, but itself having high sono-sensitivity but low photo-sensitivity. The synergistic cytotoxicity of this compound (RB2), when combined with HIFU, is better than 95%. This is well beyond anything described in the current sonodynamic literature[2,3,4]. Alone, neither the HIFU nor the RB2 show any cytotoxic effects. Animal studies to date have shown promising results in ablating superficial MDA-MB-231 (breast cancer) tumors in mice using the combination of HIFU driven cavitation and systemically administered RB2. Work remains to be done to shore up this data and make it more convincing. To translate this work, a different tumor/animal model should be tested that is more compatible with HIFU geometry. Such a study should also include consideration of the possible effect on promoting or suppressing metastatic disease, either by spreading live cancer cells (worst case), or by creating an anti-cancer immune response (best case). Prior to any clinical evaluation, a toxicity study of the new compound is also essential, although our current data do not suggest any toxicity in vitro or in vivo, where we routinely used doses of 250 mg/kg without problems. Finally, to make this a practical reality, a better means of initiating cavitation at the treatment site is required. While there are a number of technically feasible options, including high power pulses of ultrasound, injected microbubbles, or laser pulses, all of these have problems that must be addressed. When these challenges are overcome, we believe this technology could become an effective, targeted, non-invasive means of rapidly and safely ablating deep seated tumors in the breast, without side effects or radiation exposure.

References

[1] Rosenthal I, Sostaric JZ, Riesz P. Sonodynamic therapy--a review of the synergistic effects of drugs and ultrasound. Ultrason Sonochem 2004;11:349-63.
[2] Hachimine K, Shibaguchi H, Kuroki M, Yamada H, Kinugasa T, Nakae Y, Asano R, Sakata I, Yamashita Y, Shirakusa T, Kuroki M. Sonodynamic therapy of cancer using a novel porphyrin derivative, DCPH-P-Na(I), which is devoid of photosensitivity. Cancer Sci 2007;98:916-20.
[3] Liu Q, Li X, Xiao L, Wang P, Wang X, Wang Y. Study of the synergistic effect on hepatoma 22 tumor cells by focused ultrasound activation of hematoporphyrin. Journal of Ultrasound in Medicine: Official Journal of the American Institute of Ultrasound in Medicine 2008;27:57-64.
[4] Wang XB, Liu QH, Wang P, Tang W, Hao Q. Study of cell killing effect on S180 by ultrasound activating protoporphyrin IX. Ultrasonics 2008;48:135-40.